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(54) Title: DIRECTED ANTISENSE LIBRARIES

(57) Abstract

A method for making a directed antisense library against a target transcript is described. A cDNA of the target transcript is cloned in an appropriate cloning vector. Next, a plurality of deletion derivatives of the cloned cDNA is prepared such that the deletions serially extend into the cDNA from one end thereof. The resulting deletion library is then treated such that cDNA is removed from the other end of each cDNA insert, thus obtaining a fragment library having fragments of a selected size. An antisense gene is then inserted into each fragment of the fragment library, resulting in the directed antisense library. An illustrative antisense gene in the hammerhead ribozyme catalytic core. Plasmids for making the antisense library, plasmids and methods for making the fragment library, and a method for identifying target sites for antisense–mediated gene inhibition are also described.

DIRECTED ANTISENSE LIBRARIES

BACKGROUND OF THE INVENTION

This invention relates to antisense agents. More particularly, the invention relates to compositions and methods for generation of directed antisense libraries and methods of use

5   thereof wherein the antisense agents in the libraries can potentially bind to every binding site on a selected RNA transcript.

Antisense RNA, DNA, and ribozymes have been widely studied as research tools and potential therapeutic agents for inhibiting the expression of specific genes. These agents operate by binding to a complementary region on an RNA transcript produced from the gene of interest.

10   On binding, the antisense agent can prevent expression of the RNA, and this can occur through a variety of different mechanisms. There are many sites on any given RNA for targeted inhibition by an antisense molecule. For a typical RNA transcript of 2000 nucleotides, just under 2000 target sites are available. Examination of a few to tens of randomly chosen target sites reveals a great variability in activity. Clearly, not all target sites are equivalent in their ability to permit

15   antisense mediated inhibition. Consequently, identification of effective target sites on the RNA transcript for interaction with the antisense molecule is imperative for successful application of antisense technology. Methods currently available for this purpose include the use of computer algorithms to predict target accessibility based on the predicted secondary structure of the mRNA, the use of randomized oligonucleotide and ribozyme libraries in cell free systems, and

20   the examination of a few to tens of antisense oligonucleotides, targeted to arbitrarily chosen sites, in cell culture assays. These approaches have met with limited success.

To identify the most effective target site(s), the following conditions should be met. First, all possible sites on the target RNA should be evaluated. Second, evaluation should be carried out in the normal cellular milieu. This insures that the target is in its natural structure,

25   associated with its normal complement of cellular factors. Additionally, the antisense agent has the opportunity to act on alternate structures that may arise as a result of the many RNA processing reactions.

To evaluate all target sites, antisense libraries must be used. These libraries should contain antisense molecules targeted to every site. One approach is the use of completely

30   randomized DNA, RNA, or ribozyme libraries. The use of completely randomized libraries suffers from two major disadvantages. First, while such libraries may contain antisense molecules directed at all sites on the target RNA, they also contain antisense molecules directed

1

at all sites of all potential RNA transcripts produced by the cell. Therefore, these random

libraries potentially have the capability to inhibit expression of every gene in the cell. Because

of this, random libraries are limited to *in vitro* use in cell free assays. Second, the complexity of

these libraries is enormous. For example, a random library that uses 14 nucleotides to recognize

5   its target must contain at least 2.6×10^8 (i.e., 4^{14}) different members. Realistically, the size of

the library must be at least 10- to 100-fold greater in size to insure representation of all

sequences. The production and screening of such large libraries is likely beyond current

capabilities.

Herein there is described a new method for identifying optimal antisense target sites

10   against any desired RNA transcript. This is a directed library approach. In other words, this

approach uses an antisense library that targets every site on any selected RNA and only sites

present on the selected RNA. This library, therefore, does not inhibit other non-target RNA

transcripts. This approach is also an improvement over known methods because it uses

relatively small libraries. For example, a library targeting an RNA transcript of 2000

15   nucleotides, and using 14 nucleotides to recognize its target, theoretically needs 1986 members.

In practice, the library would need to be 10- to 50- times this size. At 50 times, or 99,300

members, this is still a relatively small library. These directed libraries can be used in both *in*

vitro and *in vivo* assays for the detection of effective target sites for antisense mediated gene

inhibition.

20   In view of the foregoing, it will be appreciated that a method for generating directed

antisense libraries would be a significant advancement in the art. Herein is described a method

for examining the entire length of any RNA transcript for sites that are accessible to antisense

agents. This approach allows for the localization of the most effective sites for targeting with

antisense agents.

25   BRIEF SUMMARY OF THE INVENTION

It is an object of the present invention to provide a simple and inexpensive method for

producing directed antisense libraries against any selected RNA transcript.

It is also an object of the invention to provide a method of producing directed antisense

libraries wherein such libraries contain antisense agents directed against all targets spanning the

30   entire selected RNA transcript.

It is another object of the invention to provide a method of using directed antisense

libraries for locating efficient target sites on the selected RNA transcript.

It is still another object of the invention to provide compositions for use in constructing directed antisense libraries.

It is yet another object to provide a method for making fragment libraries of a selected size of DNA fragment inserted in a cloning vector.

These and other objects can be addressed by providing a method for generating an antisense library targeted to a selected RNA transcript comprising:

(a)   preparing a double-stranded cDNA, comprising a first end, a second end, and a central site thereof, from the selected RNA transcript and cloning the cDNA in a cloning vector comprising a promoter configured such that an antisense transcript of the cDNA is synthesized upon transcription mediated by the promoter, resulting in a cloned cDNA;

(b)   creating a plurality of deletion derivatives of the cloned cDNA wherein each of the plurality of deletion derivatives has a deletion extending from the first end into the cloned cDNA such that the plurality of deletion derivatives comprises a deletion library comprising deletions extend serially into the cDNA;

(c)   reducing the size of the cDNA contained in the deletion library to a preselected size by removing a portion of the cDNA from the second end thereof to result in a fragment library;

(d)   inserting an antisense gene DNA into the central site of the cDNA in the fragment library, thereby obtaining the antisense library.

Preferred cloning vectors comprise multi-cloning sequences comprising SEQ ID NO:1 and a combination of SEQ ID NO:2 and SEQ ID NO:3. In a preferred embodiment of the invention, the deletion derivatives are created with exonuclease III resection of the cloned cDNA. The size of the cDNA contained in the deletion library is preferably reduced to a preselected size by digesting the deletion library with a type IIS restriction endonuclease. Further, inserting the antisense gene DNA into the central site of the cDNA in the fragment library preferably comprises digesting the fragment library with a type IIS restriction endonuclease, thereby creating the central site, and ligating the antisense gene DNA at the central site. A preferred antisense gene comprises a ribozyme catalytic core, more preferably, a hammerhead ribozyme catalytic core.

Another aspect of the invention relates to a method for generating a library of DNA fragments of a selected size wherein the fragments collectively span all possible sites of the selected size in a source DNA comprising a first end, a second end, and a central site thereof, comprising:

3

(a)   cloning the source DNA in a cloning vector;

(b)   creating a plurality of deletion derivatives of the cloned source DNA wherein each of the plurality of deletion derivatives has a deletion extending from the first end into the cloned DNA such that the plurality of deletion derivatives comprises a deletion library comprising deletions extend serially into the cloned DNA; and

(c)   reducing the size of the DNA contained in the deletion library to a preselected size by removing a portion of the DNA from the second end thereof to result in the library of fragments.

Still another aspect of the invention relates to a method for identifying target sites for antisense-mediated inhibition of a selected gene comprising:

(a)   constructing a directed antisense library targeted at the selected gene wherein the library is contained in a cloning vector having a promoter configured for transcribing antisense transcripts from the directed antisense library in suitable cells wherein the selected gene is expressed as a target transcript;

(b)   transforming a plurality of the suitable cells such that each of the plurality of suitable cells transcribes an antisense transcript that has access to the target transcript for potential inactivation thereof;

(c)   identifying a cell wherein an antisense transcript inactivates the target transcript; and

(d)   analyzing the antisense transcript that inactivates the target transcript and determining a target site on the antisense transcript that is associated with inactivation of the target transcript.

Yet another aspect of the invention relates to a method for identifying target sites for antisense-mediated inhibition of a selected gene comprising:

(a)   constructing a directed antisense library targeted at the selected gene wherein the library is contained in a cloning vector having a promoter configured for transcribing antisense transcripts from the directed antisense library *in vitro*;

(b)   transcribing antisense transcripts from the directed antisense library *in vitro*;

(c)   incubating the antisense transcripts with a lysate from a cell containing target transcripts transcribed from the selected gene such that antisense transcripts targeted to the target transcripts bind to such target transcripts; and

(d)   analyzing the antisense transcripts that bind the target transcript and determining a target site on the antisense transcript that is associated with binding of the target transcript.

4

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

FIGS. 1A and 1B show illustrative multi-cloning sequences (MCS's) according to an aspect of the present invention.

FIG. 2 summarizes an illustrative method for making a DNA fragment library containing 14 bp fragments according to the present invention.

FIG. 3 shows a schematic representation of a hammerhead ribozyme bound to a target substrate; the hammerhead ribozyme comprises a catalytic core that cleaves the substrate at the cleavage site indicated by the arrow and a recognition domain for binding to the substrate by base pairing.

FIG. 4 summarizes an illustrative method for making a hammerhead ribozyme library from an antisense RNA library according to the present invention.

FIG. 5A shows an illustrative method for inserting a selected cassette at an end of a deletion fragment in a deletion fragment library according to the present invention.

FIG. 5B shows an illustrative method for inserting a selected cassette in a MCS prior to preparation of a deletion fragment library according to the present invention.

FIG. 6A shows a map of expression vector pBK, which is suitable for use in identifying antisense targets in mammalian cells according to the present invention.

FIG. 6B shows base pairing of nucleotides in a multi-cloning sequence flanked by cis-acting ribozymes (CAR's).

FIG. 7A shows a map of vector pASlib according to the present invention.

FIG. 7B shows a map of vector pShuttle according to the present invention.

FIG. 7C shows a map of the MCS of pShuttle according to the present invention.

FIG. 8 shows a histogram of the distribution of 56 target sites in an illustrative antisense library according to the present invention.

DETAILED DESCRIPTION

Before the present compositions and methods for generating directed antisense libraries and methods of use thereof are disclosed and described, it is to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out herein.

5   As used herein, "antisense agent" and similar terms mean antisense RNA, antisense DNA, and ribozymes.

As used herein, "comprising," "including," "containing," "characterized by," and grammatical equivalents thereof are inclusive or open-ended terms that do not exclude additional, unrecited elements or method steps. "Comprising" is to be interpreted as including

10   the more restrictive terms "consisting of" and "consisting essentially of."

As used herein, "consisting of" and grammatical equivalents thereof exclude any element, step, or ingredient not specified in the claim.

As used herein, "consisting essentially of" and grammatical equivalents thereof limit the scope of a claim to the specified materials or steps and those that do not materially affect the

15   basic and novel characteristic or characteristics of the claimed invention.

Construction of Directed Antisense Libraries

The present invention includes a procedure that allows construction of directed antisense libraries of a variety of types. This requires the use of specially designed bacterial and/or mammalian plasmid vectors. Most importantly, these vectors possess a specially designed

20   multi-cloning sequence (MCS). This approach is not restricted to a single MCS, as many can be designed that allow the procedure to be performed. Two illustrative MCS's are shown in FIGS. 1A (SEQ ID NO:1) and 1B (SEQ ID NO:2 and SEQ ID NO:3). These simply illustrate two possible multi-cloning sequences that could be used for this method. While some of the same restriction enzyme sites are used in both of these MCS's, such particular sites are not necessarily

25   the only sites that could be used. Many other restriction enzyme sites could substitute for any of the restriction sites, allowing the same procedure to be performed.

The procedure uses a special multi-cloning sequence and a series of enzymatic manipulations to produce DNA fragment libraries directed against any desired gene of interest. The fragment libraries contain all overlapping fragments spanning the entire length of the gene

30   of interest. Transcription *in vitro* or *in vivo* of the DNA fragment allows the production of an antisense RNA targeted to the site on the RNA transcript that is encoded by the DNA fragment. Transcription of the entire DNA fragment library produces all antisense RNA molecules

6

targeting all positions on the RNA target. Expression of the library in mammalian cells allows
identification of effective target sites for antisense-mediated gene inhibition.

The procedure is illustrated in FIG. 2 using the MCS shown in FIG. 1A. Beginning with
the MCS 10 in a suitable circular plasmid vector (described in more detail below), a blunt-ended
5   DNA fragment encoding the gene of interest 14 is ligated into the EcoRV-digested MCS (FIG. 2,
step a). Since the gene can be inserted in one of two orientations, a clone is selected, according
to methods well known in the in art such as nucleotide sequencing or restriction mapping,
wherein the gene insert is suitably oriented. The orientation will depend on the placement of a
transcriptional promoter adjacent to the MCS. The orientation of the insert will be chosen such
10   that the antisense strand of the insert will be transcribed by the adjacent promoter. Next, a
deletion library is prepared. The plasmid containing the gene of interest is digested with both
PmeI and BbeI (FIG. 2, step b). The BbeI terminus is protected from exonuclease III digestion
because of its 3′ overhang 18, while the PmeI terminus 22 is a suitable substrate therefor. The
digested plasmid is then treated with exonuclease III and aliquots are removed over time into a
15   stop mixture (FIG. 2, step c). The time points are chosen such that deletions are generated after
every nucleotide across the entire gene. After exonuclease III digestion, the combined aliquots
are treated with mung bean nuclease to remove the resulting 5′ overhang (FIG. 2, step c). The
termini are then polished with T4 DNA polymerase (FIG. 2, step d) and the plasmid is re-
circularized with T4 DNA ligase to produce the deletion library (FIG. 2, step e). The deletion
20   library is then converted into a fragment library (14 base-pair fragments 26 in this case) by
digestion with restriction endonucleases BsmI and BpmI (FIG. 2, step f), purification of the
plasmid containing the 14 bp fragment 26 from the excised BpmI/BsmI fragment 30 (FIG. 2,
step g), end-polishing with T4 DNA polymerase (FIG. 2, step h), and ligation with T4 DNA
ligase (FIG. 2, step i). Not stated, but implied, after each ligation step (i.e., steps a, e, and i) the
25   ligation mixture is transformed into bacteria, the DNA is recovered from the bacteria, and the
recovered DNA is used in the subsequent step. All of these reactions involving restriction
endonucleases, ligases, polymerases, nucleases, and the like are well known in the art and are
performed according to standard methods, e.g., J. Sambrook et al., Molecular Cloning: A
Laboratory Manual (2d ed., 1989); T. Maniatis et al., Molecular Cloning: A Laboratory Manual
30   (1982); F. Ausubel et al., Current Protocols in Molecular Biology (1987), relevant parts of which
are hereby incorporated by reference.

The essence of the procedure is as follows. A gene of interest is converted into a library
of fragments serially deleted after every nucleotide. This deletion library is subsequently

converted into a fragment library containing all overlapping fragments encoded by the gene.

The fragment library can also serve as the starting point for construction of other types of antisense libraries. One such library is an antisense hammerhead ribozyme library.

A hammerhead ribozyme 34 is a small RNA that can catalyze the cleavage of a complementary RNA target 38 (FIG. 3). The hammerhead comprises a catalytic core 42 (SEQ ID NO:4), essential for cleavage activity. Additionally, the hammerhead has a recognition domain 46 that is required for interaction with a complementary substrate, such as an RNA transcript. There are few sequence requirements for the recognition domain, thus by changing the sequence of the recognition domain almost any sequence can be targeted for cleavage by the hammerhead. Cleavage of the substrate 38 occurs at a cleavage site 50 containing an NUH sequence (where N is A, C, G, or U and H is A, C, or U). In the case where the substrate is a gene transcript, the hammerhead can be used as an antisense inhibitor of gene expression.

To convert the fragment (antisense RNA) library into a hammerhead ribozyme library, a DNA fragment encoding the hammerhead catalytic core is inserted into the DNA fragment encoding the antisense RNA. This is performed as illustrated in FIG 4. The 14 base-pair fragment 54 in the fragment library is bisected with HphI (FIG. 4, step a). The resulting single-stranded overhang on each terminus is then removed using the 3' to 5' exonuclease activity of T4 DNA polymerase (FIG. 4, step b) to result in blunt ends 58, 62. A DNA fragment encoding the hammerhead catalytic core 66 (SEQ ID NO:4) is then inserted by ligation (FIG. 4, step c). The catalytic core shown in FIG. 4 is interrupted by a promoter-less chloramphenicol resistance gene 70 (CAT). A promoter is provided flanking the MCS. Transforming bacteria and selecting for chloramphenicol resistance allows selection for clones in which the catalytic core is in the correct orientation to produce a bonafide hammerhead ribozyme. Next, the CAT gene is removed and the sequence encoding a hammerhead ribozyme 74 is generated by NruI digestion (FIG. 4, step d) and ligation with T4 DNA ligase (FIG. 4, step e).

Other types of antisense libraries can also be produced from the fragment library. For instance, other cassettes can be ligated into an HphI-digested fragment library. Catalytic cores from other ribozymes, including those currently known and those to be discovered, can be inserted. Additionally, other cassettes could be used that encode sequences that cause modification to the target by mechanisms other than cleavage. Similarly, ribozyme and non-ribozyme sequences can be added to the end of the antisense sequence. This is illustrated in FIG. 5A, wherein the DNA fragment library is digested with BpmI, which digests the DNA at the distal end of the inserted fragment 78 (step a). The unpaired nucleotides resulting from this

reaction are then removed with T4 DNA polymerase (step b) to result in blunt ends 82, 86.

Next, a cassette 90 is inserted by ligation to recircularize the modified plasmid 94, now

containing the cassette inserted at an end of the insert fragment. Alternatively, instead of

inserting a cassette after the fragment library is produced, a suitable cassette can be engineered

5   into the starting multi-cloning sequence. For instance, the HphI site of the original MCS (FIG.

1A) could be replaced with a cassette encoding any desired sequence (FIG. 5B). Then, using the

same procedure illustrated in FIG. 2, the cassette can be placed against the fragment sequence in

the conversion of the deletion library into the fragment library. An example of a possible

cassette is one encoding the sequence CUGA. An antisense RNA with this sequence at its 3'

10   end has been shown to be capable of directing the 2'-O-methylation of the complementary target

(J. Cavaille et al., Targeted ribose methylation of RNA in vivo directed by tailored antisense

RNA guides, 383 Nature 732-735 (1996)). This reaction is catalyzed by modification machinery

present in mammalian cells. 2'-O-methylation of a suitable target site could be used to inhibit

expression of the RNA transcript. Other cellular RNA processing reactions can also be used in a

15   similar fashion with the use of different cassettes placed adjacent to the antisense RNA

sequence.

Use of Directed Libraries in the Identification of Target Sites for Antisense-Mediated Gene Inhibition

Antisense libraries prepared according to the present invention can be assayed *in vitro* in

20   a cell free system or *in vivo* in cultured cells, as will be described in more detail below.

In vivo assay. For *in vivo*, use the antisense library is introduced by transfection into a

suitable cell line that expresses the gene of interest. The transfection conditions are chosen such

that only one member of the library is taken up by each individual cell. The individual cells then

each express a different antisense molecule targeted to a different site on the RNA transcript of

25   interest. All target sites are represented in the entire cell population produced by transfection.

Using a suitable detection method, cell clones can be identified in which expression of the target

RNA has been reduced or eliminated. These clones possess an antisense molecule that targets an

effective site on the RNA transcript of interest. The plasmid encoding this antisense molecule is

recovered and the target sequence is identified by DNA sequencing.

30   To identify suitable targets *in vivo*, specially designed expression vectors are required.

One key feature of such expression vectors is that they are designed to replicate episomally in

mammalian cells. FIGS. 6A and 7B show two such episomal vectors, pBK (SEQ ID NO:17)

9

and pShuttle (SEQ ID NO:14), respectively. Vector pBK possesses the origin of replication and
the gene encoding the T/t antigen from the human papova virus BK (BKV). Vector pShuttle
possesses the origin of replication and the EBNA1 gene from the human Epstein-Barr virus
(EBV). These sequence elements allow each of the plasmids to replicate extrachromosomally
5   (episomally). Episomal expression is desirable for several reasons. First, it eliminates the clone-
to-clone variation in expression that occurs if stable transfectants are used. P.B. Belt et al., 84
Gene 407-417 (1989). Second, since the copy number of the episomal vector is determined
primarily by the transfection conditions and, once established, remains tightly regulated, J.L.
Yates & N. Guan, 65 J. Virol. 483-488 (1991), then effects on expression due to differences in
10   copy number are minimal. Consequently, the selection of antisense efficacy is based on
accessibility and not the level of expression. Third, the use of an episomal expression vector
allows for high transfection efficiency. P.B. Belt et al., 84 Gene 407-417 (1989); R.F.
Maragolskee et al., 8 Mol. Cell. Biol. 2837-2847 (1988). This is important to ensure that all
antisense agents present in the library are represented in the mammalian transfectants. Finally,
15   the plasmid can be recovered and shuttled back into bacterial cells. This allows the sequence of
effective antisense agents to be determined, thereby identifying accessible target sites. As a
demonstration of episomal replication, pShuttle was used to transfect HeLa cells, and the cells
were grown in culture under 400 μg/ml hygromycin selection. After 1 month in culture, low
molecular weight DNA was isolated from 1 x 10^7 cells and used to transform *Escherichia coli*
20   DH5α, producing a total of 2475 hygromycin-resistant colonies.

Vector pBK illustrates other features of value for *in vivo* expression of antisense
libraries. pBK has a single antibiotic resistance gene, bleomycin^R, driven by dual mammalian
(CMV) and bacterial (em7) promoters. This allows the same selectable marker to be used in
both bacterial and mammalian cells. This helps to minimize the size of the vector, since large
25   vectors transfect at a lower efficiency. pBK has both the BK origin of replication and the origin
of replication from the pUC series of bacterial plasmids. Therefore pBK can be replicated in
both bacterial and mammalian cells, and can be shuttled between them. pBK was designed such
that the antisense library could be constructed and expressed from the same vector. The
antisense sequence is expressed by read-through expression of the bleomycin^R gene. This
30   ensures expression of the antisense agent when the cells are grown in the presence of bleomycin.
The antisense fragment is released from the larger bleomycin transcript by the activity of cis-
acting ribozymes (CAR), hammerhead ribozymes in this case, that flank the antisense sequence.
In the absence of CAR, flanking sequences of the larger bleomycin transcript could inhibit the

activity of the antisense agent. Sequences outside of the MCS (FIG. 1A) encode the cis-acting

ribozymes. They are illustrated in FIG. 6B where only the sequence of the upper strand of the

MCS is shown (SEQ ID NO:18). On cleavage by the CAR, the antisense agent is released and

stable hairpin loops form to increase the nuclease resistance of the antisense agent.

5   pShuttle shares many of the same features as pBK, with two significant differences. First,

this episomal vector is EBV-based rather than BKV-based. The second and more significant

difference is that construction of the antisense library is not possible in pShuttle. Instead, the

antisense library is first constructed in pASlib (SEQ ID NO:7), and subsequently transferred to

pShuttle for expression in mammalian cells. The antisense encoding fragment of pASlib is

10   removed by digestion with HindIII and SalI (Figure 7A). Subsequently, the HindIII/SalI

fragment is ligated into the multi-cloning site of pShuttle via the HindIII and XhoI sites (Figure

7C). This places the antisense sequence downstream of a dual CMV/T7 promoter for expression

in vivo in mammalian cells or, alternatively, *in vitro* by transcription using T7 RNA polymerase.

Although it is believed that episomal shuttle vectors are advantageous for expression of

15   directed antisense libraries, viral vectors can also be used. Many viruses are currently being

examined for expression of foreign genes for the purpose of gene therapy. These same viral

vectors would be suitable for expression of directed antisense libraries. Some of these vectors

replicate extrachromosomally and therefore behave similarly to the described episomal vectors.

Others integrate into chromosomes. For the use of integrative viral vectors, two minor problems

20   would need to be dealt with. First, the antisense gene present within the viral vector would

integrate into the chromosome with the virus. Consequently, recovering the gene to determine

the site at which it targets is not readily possible. This can be dealt with by using polymerase

chain reaction (PCR) to amplify the integrated antisense gene. The PCR product could be

sequenced directly, or cloned and sequenced to identify the target site. Second, some of these

25   viral vectors integrate randomly and this would produce differing levels of expression from

different members of the directed antisense library. As discussed, it is important that expression

of all members of the library be comparable. This problem can be dealt with by using a viral

vector that integrates at a specific preferred site, such as adeno-associated virus.

In vitro assay. Identification of effective antisense target sites using antisense libraries

30   can also be performed using *in vitro* assays. For instance, an assay such as that used by Lieber

and Strauss (A. Lieber & M. Strauss, Selection of efficient cleavage sites in target RNAs by

using a ribozyme expression library, 15 Molecular and Cellular Biology 540-551 (1995)), can

be used. For this, the antisense library is produced by *in vitro* transcription from a suitable

11

promoter. In the present case, an antisense ribozyme library in pShuttle might be used. Of course other types of antisense libraries could be used similarly. The library-containing pShuttle is digested with XbaI and used as a template for run-off transcription of the antisense ribozyme by *in vitro* transcription with T7 RNA polymerase, according to methods well known in the art

5   (e.g., C.J. Noren et al., 18 Nucleic Acids Res. 83-88 (1990). Subsequently, the transcribed ribozyme library is incubated in a lysate prepared from a mammalian cell line expressing the gene of interest. Effective target sites are identified by performing a primer extension reaction on purified RNA from the lysate using a primer specific for the gene of interest. Primer extension products terminate at the sites of cleavage by effective ribozymes. These sites are

10   identified by gel electrophoresis of the primer extension products with suitable size markers.

Example 1

Construction of pASlib. In this example, there is described an illustrative plasmid according to the present invention for making a deletion library of a selected DNA. This plasmid was constructed as follows.

15   The HindIII-HpaI fragment of pLA2917 (J.N. Allen & R.S. Hanson, 161 J. Bact. 955-962 (1985)), containing the kanamycin resistance gene, was inserted into HindIII/SmaI-digested pUC19 to produce pUCKan. An HphI and two BsaHI sites were eliminated from the kanamycin resistance gene by site-directed mutagenesis, according to methods well known in the art, to produce pUCKan*. The mutagenized kanamycin resistance gene was removed by

20   HindIII/EcoRI digestion, and the termini were blunted by 5'-overhang fill-in using the Klenow fragment of DNA polymerase I and ligated to the 843 bp BspHI-SapI fragment of pUC19 containing the origin of replication. A clone (pKan) was selected wherein the EcoRI and BspHI sites were juxtaposed. The BsmFI and PstI sites were eliminated from pKan by site-directed mutagenesis using the procedure of E. Merino et al., 12 Biotechniques 508-510 (1992). The

25   multiple cloning site for pASlib was constructed from the overlapping oligodeoxynucleotides MCS-L (SEQ ID NO:5) and MCS-R (SEQ ID NO:6) by 5'-overhang fill-in with the Klenow fragment of DNA polymerase I. Oligonucleotides were synthesized using an Applied Biosystems automated oligonucleotide synthesizer. The double-stranded multiple cloning site was inserted into EcoRI-linearized and blunted pKan to result in pASlib (SEQ ID NO:7).

30   Restriction endonuclease digestions, primer extension reactions, ligation reactions, and the like were carried out according to methods well known in the art. E.g., J. Sambrook et al., Molecular Cloning: A Laboratory Manual (2d ed., 1989); T. Maniatis et al., Molecular Cloning: A

Laboratory Manual (1982); F. Ausubel et al., Current Protocols in Molecular Biology (1987).

Therefore, pASlib possesses the pUC19 origin of replication and a kanamycin resistance gene allowing selection in bacterial cells. The kanamycin resistance gene was chosen as the selectable marker since, of all the available bacterial selection markers, it possessed the fewest

5   sites present in the MCS. Therefore, it was the simplest to modify by site-specific mutagenesis to eliminate the undesirable sites. The MCS contains the following salient features. It possesses a short polylinker that allows much flexibility in the cloning of the gene or cDNA sequence of interest, which represents the first step in the construction of an antisense library. The polylinker includes several restriction sites that leave sticky ends upon digestion. These sites can be used to

10 directionally clone the cDNA or genomic fragment in the correct orientation. Alternatively, the fragment can be cloned by blunt-end ligation, and the correctly oriented clone can be selected by restriction analysis. The PstI and PmeI sites allow the generation of a substrate for unidirectional digestion by exonuclease III into the cloned cDNA or genomic fragment. This allows preparation of a serial deletion library of the cloned insert. The BsmFI and BbsI sites are

15 used together to convert the deletion library into a 14 bp fragment library. The HphI site allows bisection of the 14 bp fragment library for introduction of the antisense agent.

Example 2

Construction of pShuttle. In this example, there is described the construction of an illustrative plasmid for use according to the present invention for expressing an antisense agent

20 in either mammalian cells using the intermediate-early promoter from cytomegalovirus or *in vitro* using T7 polymerase.

A hygromycin expression cassette capable of being expressed in both mammalian and prokaryotic systems was constructed using overlap extension PCR. PCR was carried out according to methods well known in the art, e.g., U.S. Patent No. 4,683,195; U.S. Patent No.

25 4,683,202; U.S. Patent No. 4,800,159; U.S. Patent No. 4,965,188; PCR Technology: Principles and Applications for DNA Amplification (H. Erlich ed., Stockton Press, New York, 1989); PCR Protocols: A guide to Methods and Applications (Innis et al. eds, Academic Press, San Diego, Calif., 1990). The 1026 bp hygromycin resistance coding sequence from EBOpLPP (ATCC) was joined at its 3'-end to the 322 bp 3'-untranslated region (UTR)/SV40 early polyadenylation

30 sequence from pRC/CMV (Invitrogen Corp., Carlsbad, California), while the 527 bp dual ampicillin/SV40 early promoter from pEGFP-1 was joined to the 5'-end. The sequences of the primers used in the PCR were as follows: 3'-UTR/poly(A) segment, SEQ ID NO:8 and SEQ ID

13

NO:9; hygromycin coding region, SEQ ID NO:10 and SEQ ID NO:11; amp/SV40 early

promoter, SEQ ID NO:12 and SEQ ID NO:13. Each portion of the hygromycin cassette was

prepared by PCR using one of the three primer sets and the appropriate template. The resulting

fragments were gel purified. The hygromycin-encoding and the 3'-UTR/poly(A) fragments were

5   combined and used in a second PCR reaction to produce the hygromycin-3'-UTR/poly(A)

fragment. In a final PCR, this fragment was combined with the amp/SV40 fragment to produce

the complete 1875 bp hygromycin gene cassette. The hygromycin gene cassette was ligated into

the 843 bp BspHI-SapI oriP-containing fragment of pUC19, producing pHyg. The 4914 bp

EcoRI-BamHI fragment containing the EBNA-1 and EBV oriP sequences from EBOpLPP was

10   inserted between the hygromycin cassette and the pUC19 origin of XhoI-digested pHyg to make

pEBV. The 1060 bp expression cassette was excised from pRC/CMV using NruI and PvuII and

inserted into the BamHI site of pEBV to produce pShuttle (SEQ ID NO:14).

pShuttle was designed to allow replication and expression of the antisense library in

mammalian cells. It possesses an MCS for insertion of the antisense library. The MCS is

15   flanked on one end by a dual CMV/T7 promoter for allowing expression of the antisense agent

gene both in mammalian cells as well as by *in vitro* transcription using T7 RNA polymerase. On

the other end of the MCS is a bovine growth hormone polyadenylation signal for efficient

expression in mammalian cells. pShuttle possesses a hygromycin resistance gene driven by a

dual promoter for allowing selection in bacterial and mammalian cells. The pUC19 origin of

20   replication allows replication in bacterial cells. For replication in mammalian cells, the EBV

origin and EBNA-1 gene were included. J. Yates et al., 81 Proc. Nat'l Acad. Sci. USA 3806-

3810 (1984); J. Yates et al., 313 Nature 812-815 (1985).

Example 3

Construction of a hammerhead ribozyme catalytic core cassette. A cassette encoding the

25   hammerhead catalytic core, interrupted by the CAT gene (S. Horinouchi & B. Weisblum, 150 J.

Bact. 815-825 (1982)), was constructed as follows. PCR primers were prepared that were

complementary to the CAT gene on their 3'-ends and encoded the hammerhead catalytic core on

their 5'-ends. The sequences of the primers were SEQ ID NO:15 and SEQ ID NO:16. Located

between the CAT and hammerhead catalytic core sequences were NruI restriction sites. The

30   PCR contained 5 ng CAT gene DNA, 100 pmol each of the primers CatCass 1 and CatCass 2, 1

mM of each of the four dNTPs, 5 units of VENT polymerase (New England Biolabs, Beverly,

Massachusetts) in the standard VENT polymerase buffer except that the concentration of the

MgSO$_4$ was increased to 5.2 mM (i.e., 10 mM KCl, 10 mM (NH$_4$)$_2$SO$_4$, 20 mM Tris-HCl (pH 8.8 at 24°C), 5.2 mM MgSO$_4$, 0.1% Triton X-100). The use of VENT polymerase ensured that the cassette possessed blunt ends.

The reaction mixture was incubated as follows:

5   (A)   2 minutes at 94°C;

(B)   5 cycles of 1 minute at 94°C, 30 seconds at 45°C, and 2 minutes at 72°C;

(C)   15 cycles of 30 seconds at 94°C, 15 seconds at 60°C, and 2 minutes at 72°C; and

(D)   5 minutes at 73°C.

After amplification, the cassette was purified from unincorporated primers by agarose gel electrophoresis, and the agarose was subsequently removed from the cassette DNA using standard procedures.

Introduction of a catalytic core into a fragment library presents several difficulties. The core must be inserted by blunt-end ligation and in the correct orientation to produce a functional ribozyme. Additionally, due to its small size, it is difficult to prevent the introduction of concatamers of the core and/or contamination of the library with clones that do not acquire a catalytic core. To increase the effectiveness and efficiency of this step, the core interrupted by the CAT gene was designed. CAT selection allows the use of a non-phosphorylated cassette. This prevents insertion of multimers and selects against non-recombinants. Additionally, the CAT gene allows selection of clones acquiring a correctly oriented catalytic core. In the desired orientation, transcription of the CAT and kanamycin genes is in the same direction. In the incorrect orientation, CAT expression is inhibited by antisense expression from the kanamycin resistance gene. This phenomenon has been noted previously, R. Bruckner et al., 32 Gene 151-1160 (1984). After selection, the CAT gene is removed by digestion with NruI to produce a sequence encoding a hammerhead ribozyme.

Example 4

Construction of a Herpes ICP4 ribozyme library. The 4489 bp BglII-EcoRI fragment from pTEG2, X.X. Zhu et al., 184 Virology 67-78 (1991), containing a herpes simplex virus ICP4 genomic fragment was cloned into EcoRI-EcoRV-digested pASlib. This fragment included 125 bp upstream of the translational start site, 466 bp downstream of the translational termination sequence, and the entire genomic coding sequence of ICP4. The resulting clone, pASlib-ICP4, contained the ICP4 fragment with the sense strand as the upper strand.

From pASlib-ICP4 a deletion library was produced as follows. Twenty μg of CsCl

15

gradient purified plasmid DNA was digested with *Pst*I and *Xba*I, then concentrated and desalted using a Microcon 50 spin filter (Amicon). The DNA was brought up to a volume of 60.4 μl of exonuclease III buffer (i.e., 50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 10 mM 2-mercaptoethanol), warmed to 37°C, and then 300 units of exonuclease III were added. At 1-minute intervals after

5   the addition of the exonuclease III, 2.5 μl of the reaction mixture was removed and placed in microfuge tubes on ice containing 7.5 μl of 66.7 mM sodium acetate, pH 5.2, 200 mM NaCl, 1.3 mM ZnCl$_2$, and 1 unit of mung bean nuclease. After 25 aliquots had been removed, the mung bean nuclease-containing tubes were incubated at 20°C for 30 minutes. After 30 minutes, the contents of all of the mung bean nuclease-containing tubes were combined and extracted with

10   phenol-chloroform (50:50), extracted with chloroform, and then were precipitated with two volumes of 100% ethanol. The DNA was then pelleted and dried. The DNA was then resuspended in 18 μl of d.i. H$_2$O, 2.5 μl of 10 mM of each of the four dNTPs, 2.5 μl of 10x *Pfu* polymerase buffer (e.g., 100 mM KCl, 100 mM (NH$_4$)$_2$SO$_4$, 200 mM Tris-Cl (pH 8.75), 20 mM MgSO$_4$, 1% Triton® X-100, 1000 mg/ml BSA), and 5 units of *Pfu* polymerase (Stratagene, La

15   Jolla, California), then incubated at 72°C for 15 minutes, and cooled to room temperature. The plasmid DNA was recircularized by ligating in a large volume (1.25 ml) in a buffer containing 5% PEG for 4 hours at room temperature. Except for the modifications indicated, all ligations were performed with T4 DNA ligase under the conditions suggested by the manufacturer.

After transformation into *E. coli* DH5α, the cells were grown in liquid culture to amplify

20   the deletion library. The library DNA was purified and digested with *Bsm*FI and *Bbs*I, and then the ends were blunted with *Pfu* polymerase as described above. The DNA was then recircularized by ligation in a 600 μl volume in a buffer containing 5% polyethylene glycol (PEG) at room temperature for 4 hours. After transformation into *E. coli* DH5α, amplification and plasmid purification, 1 μg of library plasmid DNA was then subjected to digestion with 8

25   units of HphI for 1 hour at 37°C, and the ends were polished with T4 DNA polymerase. The hammerhead core cassette was inserted by ligating 0.5 μg of the HphI-digested library DNA with 5 μg of the ribozyme core sequence cassette prepared according to the procedure of Example 3. That ligation product was transformed into DH5α and grown in culture under chloramphenicol selection. After purification, 2 μg of the DNA was digested with HindIII and

30   SalI, and the terminal phosphates were removed using shrimp alkaline phosphatase (Amersham, Arlington Heights, Illinois). The HindIII/SalI digest was fractionated on an agarose gel, and the dephosphorylated ribozyme/chloramphenicol cassette was purified using standard procedures. The cassette was combined with an equimolar amount of HindIII-XhoI-digested pShuttle,

prepared according to the procedure of Example 2, and ligated using a modified two-step ligation procedure, S. Damak & D.W. Bullock, 15 Biotechniques 448-450 (1993) (hereby incorporated by reference). The first step was performed at room temperature for 1 hour, and the second step was incubated overnight at 16°C. The ligation mixture was transformed in DH5α

5   and grown in culture under chloramphenicol selection. The library DNA was purified and then digested with NruI to release the chloramphenicol gene. The digested DNA was then recircularized by ligation in a volume of 600 μl. The final ligation product was transformed into DH5α, and the plasmid DNA was purified on a CsCl gradient.

To verify the effectiveness of this procedure, 56 clones obtained at various steps were

10   sequenced. Thirty-one were from the final ribozyme library, and the remainder were from earlier steps, beginning with the 14 bp fragment library. The results of the sequencing are discussed below.

One observation made after the mung bean digestion was that the deletions infrequently stopped at A-T base pairs. While exonuclease III has been shown to exhibit a preference for

15   stopping at certain nucleotides (C>A=T>G), W. Linxweiler & W. Horz, 10 Nucleic Acids Res. 4845-4859 (1982), this was not believed to be the cause of the observed sequence bias. Instead, it is believed that this is the result of a greater degree of "breathing" at A-T terminated deletions and the subsequent removal of A-T terminal pairs by mung bean nuclease. The mung bean nuclease digestion was later performed at higher salt concentrations (150 mM) and at a lower

20   temperature (20°C). This eliminated the under-representation of A-T terminated deletions.

For construction of the library, two type IIS restriction enzymes are required, BsmFI and HphI. Typical of type IIS restriction enzymes, BsmFI and HphI cleave downstream of their recognition sequences in a sequence-independent manner. Cleavage by type IIS restriction enzymes can pose some problems since they can exhibit infidelity in how far from their

25   recognition site they cleave. Cleavage by BsmFI was largely at the expected distance (10/14), but also at 11/15. The reported 9/13 activity for this enzyme, V.E. Velculescu et al., 270 Science 484-487 (1995), was not seen in any of the clones sequenced. Infidelity of BsmFI does not present a problem for construction of ribozyme libraries. The result of this infidelity is that the recognition domains of the ribozymes in the library can vary from 13 to 15 nucleotides.

30   In contrast, HphI infidelity can be problematical. HphI digestion is a critical step in the construction of ribozyme libraries. This enzyme produces a 1 nucleotide 3'-overhang that is later removed by polishing with T4 DNA polymerase. It is essential to the proper functioning of the resulting ribozyme that this 1 nucleotide be removed, since it does not have an antisense

17

binding partner in the ribozyme (FIG. 1, X).

HphI cleaves at 8/7, but also at 9/8. D. Kleid et al., 73 Proc. Nat'l Acad. Sci. USA 293-297 (1976). This infidelity is demonstrated in the present library by the presence of ribozymes with flanking helices of length 8 and 5, as would be expected if HphI cleaved at 9/8. This type

5   of infidelity, in itself, is not problematical. It simply alters the relative lengths of the two arms of the binding domain, leaving the total length of the binding arms unchanged. The problem that arises with HphI infidelity is that the enzyme can cleave twice at the same target, i.e., if it first cleaves at 9/8 it can rebind and cleave at 8/7. The result is that 2 bp are removed from the sequence upon polishing with T4 DNA polymerase. Removal of 2 bp from the insertion site of

10   the ribozyme core produces a non-functional ribozyme. In early attempts to produce a library, >40% of the clones were the product of double cutting. This is close to the statistically predicted 50% that would occur if HphI has no preference for either 8/7 or 9/8 cutting. To minimize the possibility of double cutting, HphI digestion was performed under near "single hit" conditions. Under these conditions, double cleavage was only 13% of the final library. It should be possible

15   to further reduce the percentage of double hits by performing the cleavage under "sub-single hit" conditions. This should not present any problem so long as the amount of plasmid digested is sufficient to allow full representation of the ribozyme library. Undigested molecules cannot accept the catalytic core and are removed in the later step by selection for chloramphenicol resistance. Other class IIS restriction enzymes, such as MboII, could likely substitute for HphI,

20   but fidelity may not be any better.

The infidelity of HphI raises another issue. It is possible that some sequences favor digestion at 8/7 and others at 9/8. This could lead to the absence of some ribozyme target sequences in the final library. This appears to be unlikely, however. First, as discussed, under conditions that give nearly 100% cleavage by HphI, >40% of the molecules are cut twice. This

25   is close to the 50% predicted if HphI exhibits no preference for 8/7 versus 9/8 cutting. Second, two clones that both contain the same 14 bp sequence of ICP4, Rz8 and Rz9 (Table 1), are the products of 8/7 and 9/8 cleavage, respectively. This suggests that the intervening sequence between the binding site and the cleavage site does not affect where HphI cleaves.

HphI is also sensitive to overlapping dam methylation. This is also true of MboII. Since

30   2 nucleotides of the four base consensus sequence for dam methylation are provided by the variable sequence of the cDNA insert, mathematically 1/16 of the clones in the 14 bp fragment library (6.25%) will not be cleaved with HphI and will be eliminated from the final ribozyme library. This can be prevented by passage of the 14 bp fragment library in a dam⁻ strain prior to

18

HphI digestion.

Table 1		
Clone (Position)	Target Sequence[a]	SEQ ID NO:
Rz1 (1754)	cgacgccgcccgcc	19
Rz2 (1992)	cugcgcgcguggcu	20
Rz3 (2045)	gcgccugcgcgggg	21
Rz4 (2252)	cgccgccgacgcgc	22
Rz5 (2411)	cccccucccccgcg	23
Rz6 (2517)	guggcccugucgcg	24
Rz7 (2590)	gccacacggcggcg	25
Rz8 (2729)	cgccgcgcggugcg	26
Rz9 (2729)[b]	cgccgcgcggugcg	27
Rz10 (2837)	cccccugcgcgccuc	28
Rz11 (2915)	gguggugcuguacuc	29
Rz12 (3246)	gggcccgcgguguc	30
Rz13(3275)[c]	ccuggcgugcgagc	31
Rz14 (3569)	ggggaccaccgacgccauggc	32
Rz15 (3680)	cguggcgcuggggc	33
Rz16 (3842)	cgggauucgcuggg[d]	34

a   The nucleotide in bold indicates the unbound nucleotide, i.e., position X in FIG. 3.
b   Clone repeated 2 times.
c   Clone repeated 3 times.
d   Bona fide ribozyme target.

The target locations of the 56 sequenced clones are illustrated in FIG. 8. The histogram indicates that the target sites are fairly evenly distributed across the entire ICP4 gene, with the exception that no clones were identified targeting the 5'- and 3'-termini. It is unlikely that the library is devoid of members targeting these regions since the libraries are prepared with complexities far exceeding the total number of sites on the gene. It is even possible that target sites in these regions are similarly represented as those identified by the sequenced clones. Due to the small number of clones sequenced, it is likely that some larger gaps in the data could be observed even for a uniformly represented library, such as the gap between positions 966 and 1282.

19

Of the 56 sequences determined, 42 (75%) occurred only once, while four occurred

multiple times (FIG. 8). Three were only mildly over-represented, with two or three occurrences

compared with the single occurrence for the majority of clones. The three positions were 2054

and 3246, with two occurrences each, and position 2729, with three occurrences. One position,

5   3275, was significantly over-represented, occurring seven times. Five of the occurrences were

observed within the 32 clones sequenced from the final library, and the other two were found at

early stages of the construction. The over-representation of particular sites is likely caused by

some local sequence and/or structure in the DNA that either stalls exonuclease III or causes it to

fall off the template. P. Abarzua & K.J. Marians, 81 Proc. Nat'l Acad. Sci. USA 2030-2034

10   (1984). Performing the exonuclease III deletion at higher temperatures might reduce this

phenomenon if an inhibitory structure is forming at certain sequences. Higher temperature also

allows for more distributive activity from the enzyme, J.D. Hoheisel, 209 Anal. Biochem. 238-

246 (1993), which is desirable in this type of exonuclease III digestion. While it is possible that

the exonuclease III digestion conditions may need to be optimized for each target cDNA,

15   creating libraries larger than would be necessary to represent every position would ensure

complete representation of all target sites.

Examination of the 31 clones obtained from the final library allowed determination of the

overall effectiveness of the procedure. All 31 possessed a catalytic core, demonstrating the

effectiveness of the use of CAT selection for this purpose. Nineteen of the 31 clones *\(61%)

20   contained sequences that could potentially be ribozymes if the sequence that they targeted

included the required NUH sequence at the correct location. These are shown in Table 1.

Counted among these potential ribozymes were three clones that possess non-detrimental

defects. One has a single nucleotide deleted from loop II of the ribozyme (Rz13). This produces

a three-, instead of a four-nucleotide loop II. The site of this defect is the NruI site used to

25   remove CAT from the catalytic core. The ends must have been damaged during this step for this

clone. The other two non-detrimental defects were the result of incomplete digestion by BsmFI.

These clones have a longer flanking arm corresponding to helix III (Rz12 and Rz14). This

appears to be the result of a lack of cleavage of the BsmFI site on pASlib and, instead, an

internal BsmFI site on ICP4 was used. These clones would be expected to produce functional

30   ribozymes had they targeted an NUH sequence.

The remaining 12 clones (39% of 31) possessed defects that would prevent them from

being potentially functional ribozymes. Four of these (13%) were defective in that they were

cleaved twice with HphI. As discussed above, it is likely that this defect can be reduced to close

to zero by performing the HphI digestion under "sub-single hit" conditions. Three (9.7%) were missing 1 nucleotide from one end of the catalytic core. Since the deletion always occurred at the same end of the cassette and the thermostable polymerase used to make the cassette does not contain any 5' to 3' exonuclease activity, the PCR primer constituting that end of the cassette

5   must have been contaminated with a small percentage of a failure fragment of the DNA synthesis. This defect can be eliminated by better purification of the primers. Five clones (16%) possessed the catalytic core in the incorrect orientation. This is in contrast to the expected 50% if there were no selection for orientation. Incorrectly oriented clones could be eliminated by moving the promoter for the CAT gene outside the MCS of pASlib. Finally, three clones were

10   the result of various unknown cloning artifacts.

Therefore, the success rate of this library was 61%. As discussed, a few procedural changes would increase the success rate to 70-80%. This could be increased a further 16% by placing the CAT promoter outside the MCS. Even at 61%, the success rate is more than adequate. This just means that it is necessary to screen an antisense library 140% the size

15   needed if 100% success were achieved. This would still be a small library relative to a non-directed library approach.

Three of the 31 clones (9.7%) targeted a site on the ICP4 mRNA that contained a uridine at the proper position of the consensus NUH site (Rz3, Rz5, and Rz16). Of the three, only one targeted a consensus NUH site (Rz16). Due to the unusually high G/C content of the ICP4

20   genomic fragment used to make the ribozyme library, only 9.2% of the nucleotides in the mRNA are uridines, of which 203 occur as an NUH triplet. The fact that the percentage of sequenced clones in the library targeting an NU site is virtually identical to the percentage of uridines in the ICP4 gene suggests that the library is unbiased and likely to contain a fairly uniform distribution of target sites.

25   The use of a direct library for target site selection significantly simplifies the screening process, since only very small libraries need be prepared and assayed. For ICP4, assuming the library contains a uniform distribution of the 4475 distinct sequences (4489-14), a library of 67,125 (15-fold excess) is expected to have a probability of 99.9% of containing all sequences. W. Feller, An Introduction to Probability Theory and Its Applications (3d ed. 1968). Based on a

30   χ^2 goodness-of-fit analysis of the 56 sequences, the multiples observed at positions 2729 and 3275 occur with a higher frequency than would be expected for a uniform distribution. All other positions are consistent with a uniform distribution. Correcting for the two over-represented sequences, a library of 81,057 (18-fold excess) is expected to contain all sequences with

21

probability of 99.9%. Preparation, manipulation, and screening of such a library is well within

the limitations of current practice. In contrast, a non-directed library targeting 14 nucleotides

would require a minimum size of 2.7 x 10^8 (4^{14}). The ability to prepare and screen such a library

is questionable. Even if possible, the vast majority of members of the library are directed at non-

5   target genes. Inhibition of non-target genes could pose problems in interpreting the results.

CLAIMS

We claim:

1.   A method for generating an antisense library targeted to a selected RNA transcript comprising:

(a)   preparing a double-stranded cDNA, comprising a first end, a second end, and a central site thereof, from the selected RNA transcript and cloning the cDNA in a cloning vector comprising a promoter configured such that an antisense transcript of the cDNA is synthesized upon transcription mediated by the promoter, resulting in a cloned cDNA;

(b)   creating a plurality of deletion derivatives of said cloned cDNA wherein each of said plurality of deletion derivatives has a deletion extending from said first end into the cloned cDNA such that the plurality of deletion derivatives comprises a deletion library comprising deletions extend serially into the cDNA;

(c)   reducing the size of the cDNA contained in the deletion library to a preselected size by removing a portion of the cDNA from the second end thereof to result in a fragment library;

(d)   inserting an antisense gene DNA into the central site of the cDNA in the fragment library, thereby obtaining the antisense library.

2.   The method of claim 1 wherein said cloning vector comprises a multi-cloning sequence represented by SEQ ID NO:1.

3.   The method of claim 1 wherein said cloning vector comprises a multi-cloning sequence comprising SEQ ID NO:2 and SEQ ID NO:3.

4.   The method of claim 1 wherein said plurality of deletion derivatives is created with exonuclease III resection of the cloned cDNA.

5.   The method of claim 1 wherein said reducing the size of the cDNA contained in the deletion library to a preselected size comprises digesting the deletion library with a type IIS restriction endonuclease.

6.   The method of claim 1 wherein said inserting an antisense gene DNA into the central site of the cDNA in the fragment library comprises digesting the fragment library with a type IIS restriction endonuclease, thereby creating said central site, and ligating the antisense gene DNA at the central site.

7.   The method of claim 1 wherein said antisense gene DNA comprises a ribozyme catalytic core.

8.   The method of claim 1 wherein said ribozyme catalytic core is a hammerhead

23

ribozyme catalytic core.

9.   A method for generating a library of DNA fragments of a selected size wherein said fragments collectively span all possible sites of the selected size in a source DNA comprising a first end, a second end, and a central site thereof, comprising:

5
    (a)   cloning the source DNA in a cloning vector;

    (b)   creating a plurality of deletion derivatives of said cloned source DNA wherein each of said plurality of deletion derivatives has a deletion extending from said first end into the cloned DNA such that the plurality of deletion derivatives comprises a deletion library comprising deletions extend serially into the cloned DNA;

10
    (c)   reducing the size of the DNA contained in the deletion library to a preselected size by removing a portion of the DNA from the second end thereof to result in the library of fragments.

10.   The method of claim 9 wherein said cloning vector comprises a multi-cloning sequence represented by SEQ ID NO:1.

15
11.   The method of claim 9 wherein said cloning vector comprises a multi-cloning sequence comprising SEQ ID NO:2 and SEQ ID NO:3.

12.   The method of claim 9 wherein said plurality of deletion derivatives is created with exonuclease III resection of the cloned DNA.

13.   The method of claim 9 wherein said reducing the size of the DNA contained in

20   the deletion library to a preselected size comprises digesting the deletion library with a type IIS restriction endonuclease.

14.   The method of claim 9 wherein said source DNA is a double-stranded cDNA prepared from an RNA transcript.

15.   pASlib (SEQ ID NO:7).

25
16.   pShuttle (SEQ ID NO:14).

17.   pBK (SEQ ID NO:17).

18.   A multi-cloning sequence represented by SEQ ID NO:1.

19.   A multi-cloning sequence comprising SEQ ID NO:2 and SEQ ID NO:3.

20.   A method for identifying target sites for antisense-mediated inhibition of a

30   selected gene comprising:

    (a)   constructing a directed antisense library targeted at said selected gene wherein said library is contained in a cloning vector having a promoter configured for transcribing antisense transcripts from said directed antisense library in suitable cells wherein the selected

24

gene is expressed as a target transcript;

(b)   transforming a plurality of said suitable cells such that each of said plurality of suitable cells transcribes an antisense transcript that has access to the target transcript for potential inactivation thereof;

5   (c)   identifying a cell wherein an antisense transcript inactivates the target transcript; and

(d)   analyzing the antisense transcript that inactivates the target transcript and determining a target site on the antisense transcript that is associated with inactivation of the target transcript.

10   21.   A method for identifying target sites for antisense-mediated inhibition of a selected gene comprising:

(a)   constructing a directed antisense library targeted at said selected gene wherein said library is contained in a cloning vector having a promoter configured for transcribing antisense transcripts from said directed antisense library *in vitro*;

15   (b)   transcribing antisense transcripts from said directed antisense library *in vitro*;

(c)   incubating said antisense transcripts with a lysate from a cell containing target transcripts transcribed from said selected gene such that antisense transcripts targeted to the target transcripts bind to such target transcripts; and

(d)   analyzing the antisense transcripts that bind the target transcript and determining

20   a target site on the antisense transcript that is associated with binding of the target transcript.

1/8

Fig. 1A

Fig. 1B

2/8

Fig. 2

Fig. 3
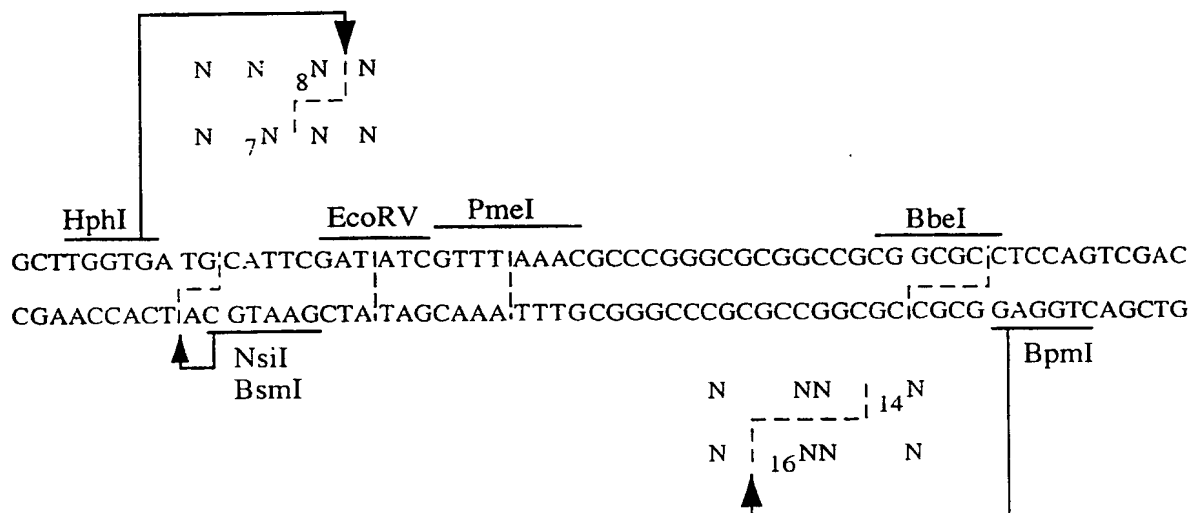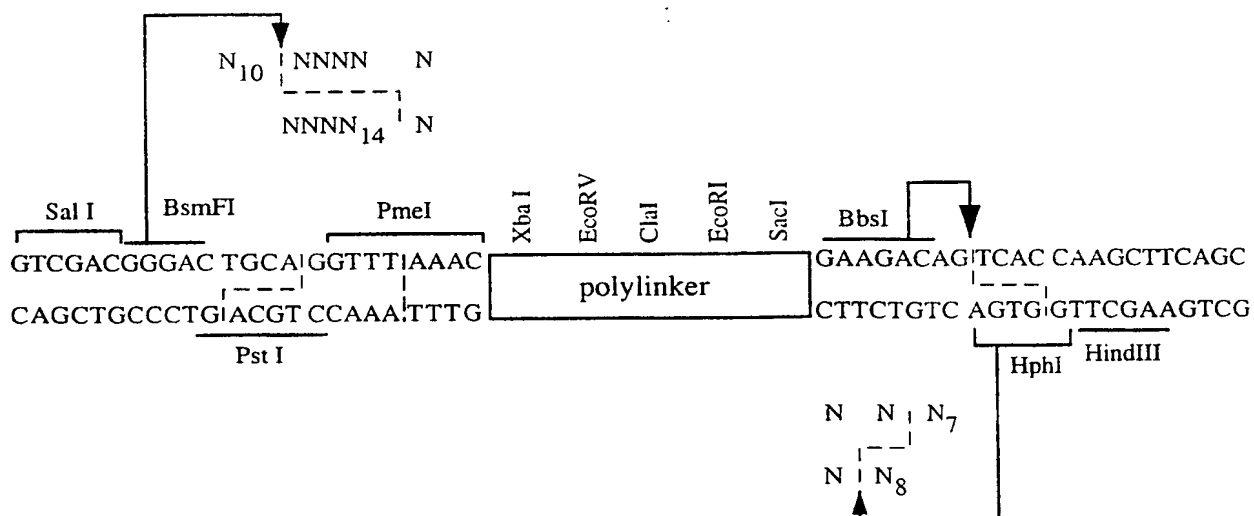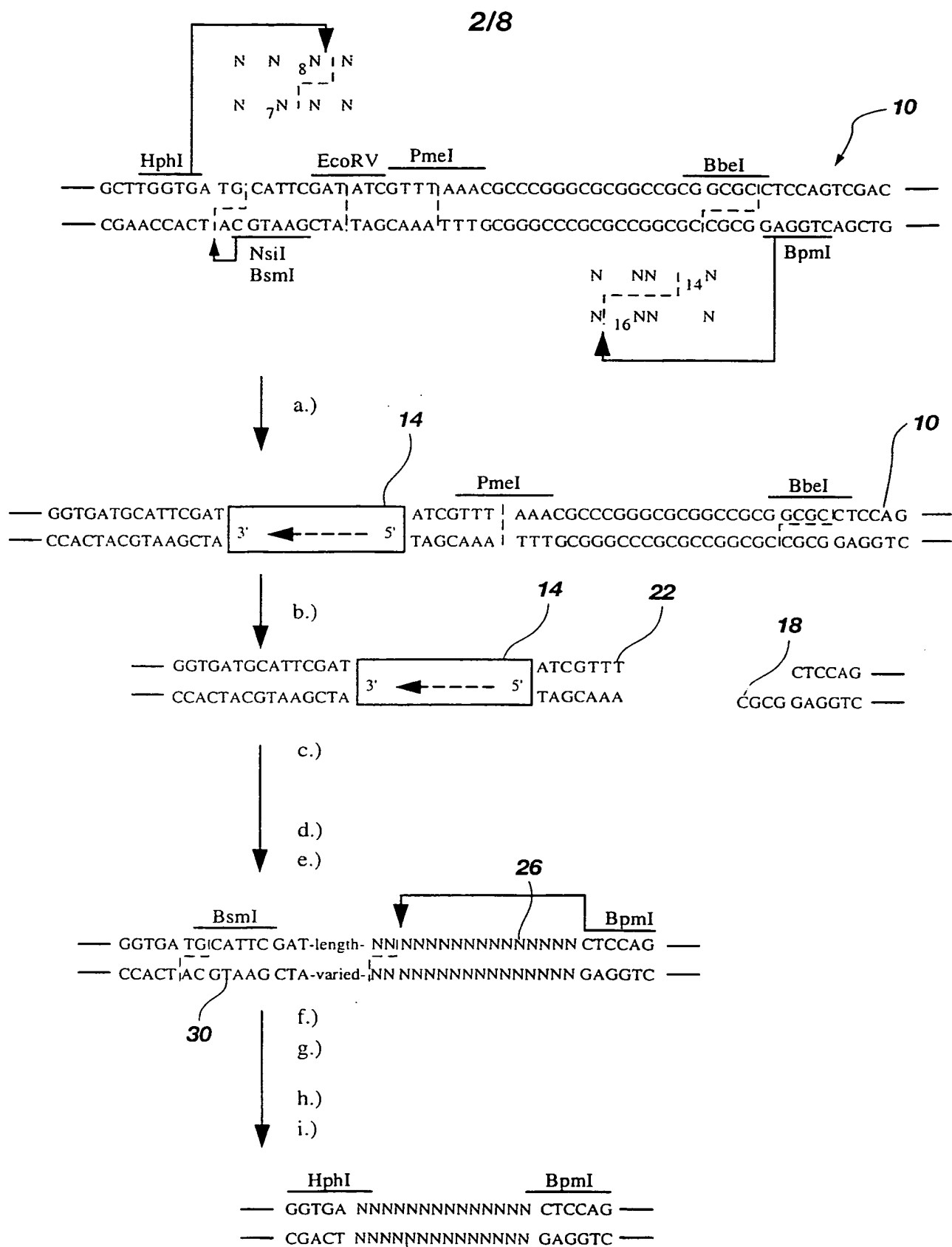
4/8

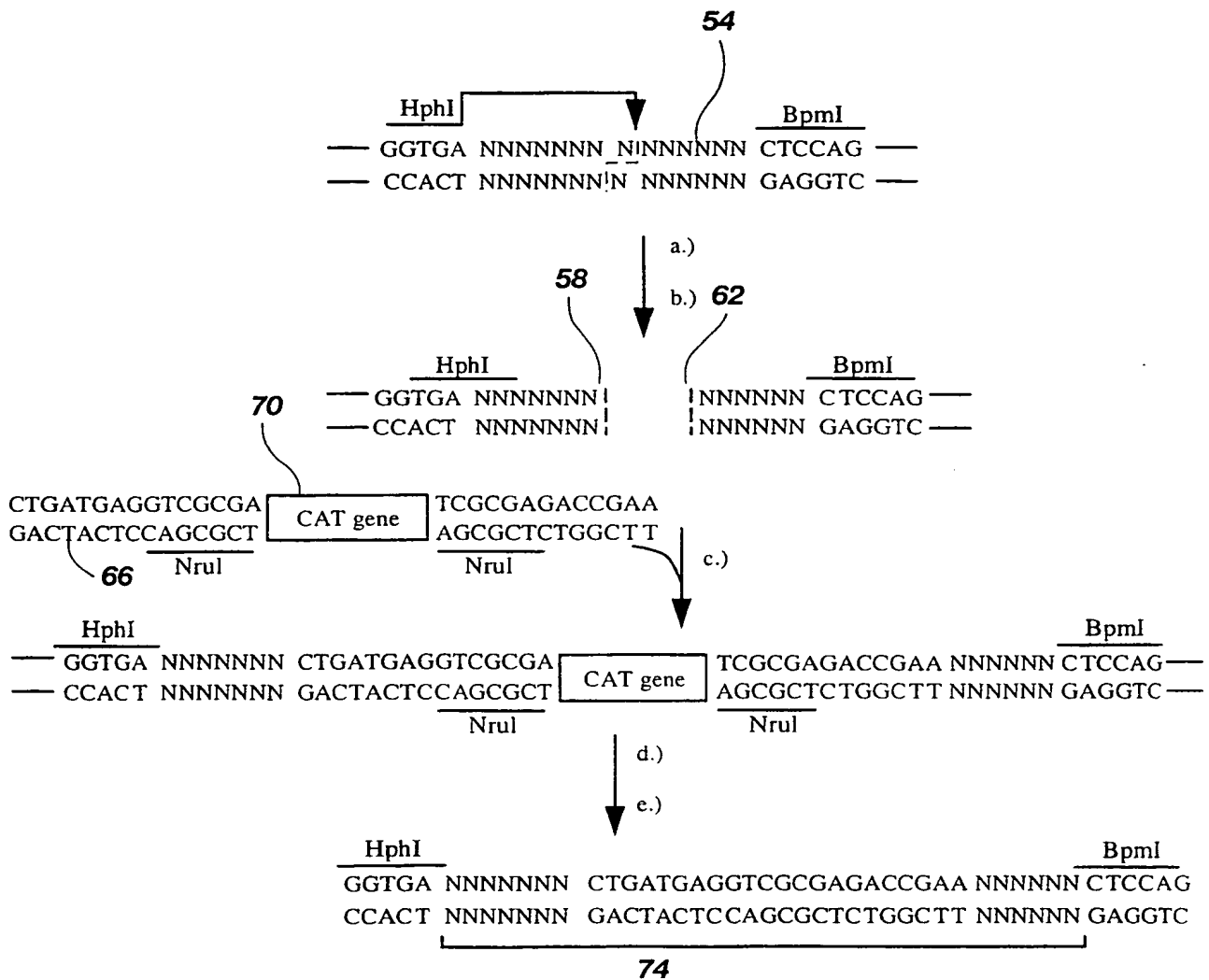

Fig. 4

5/8

Fig. 5A

Fig. 5B

SUBSTITUTE SHEET (RULE 26)
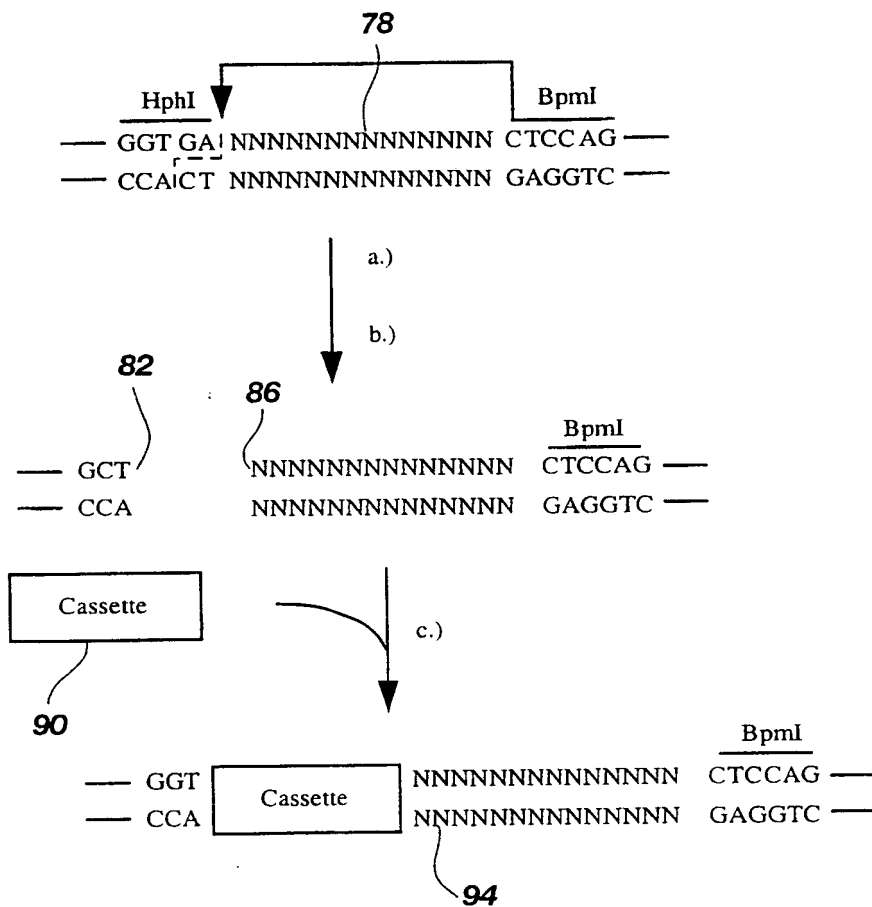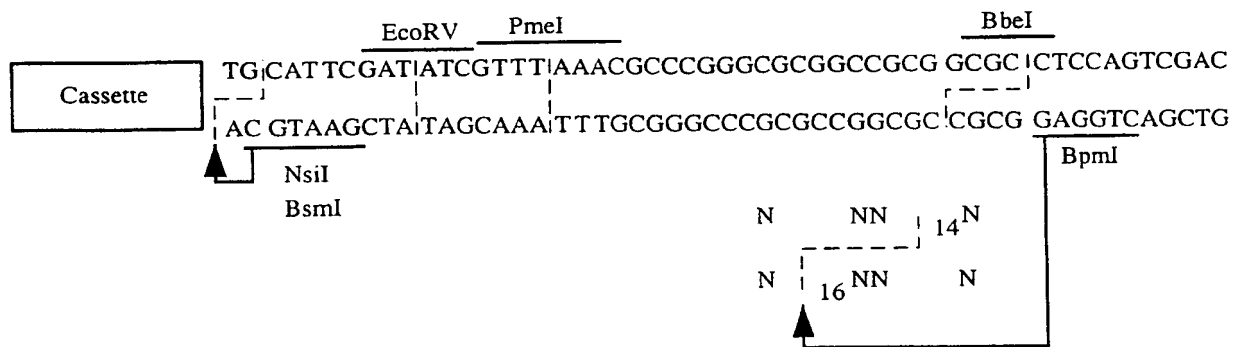
6/8
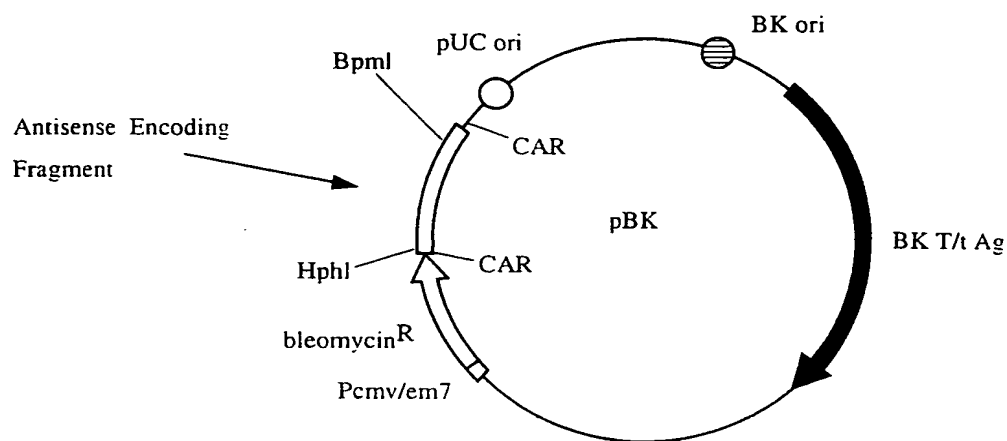
Fig. 6A
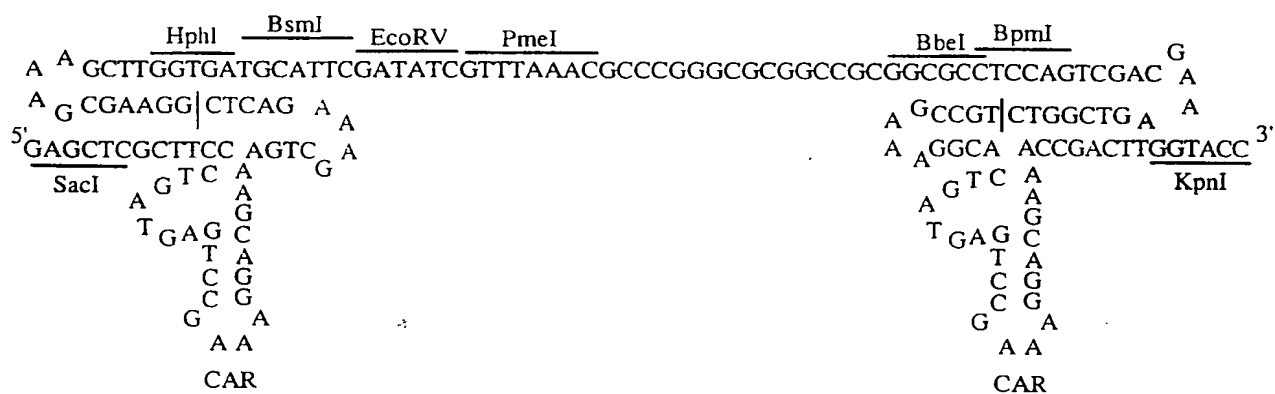
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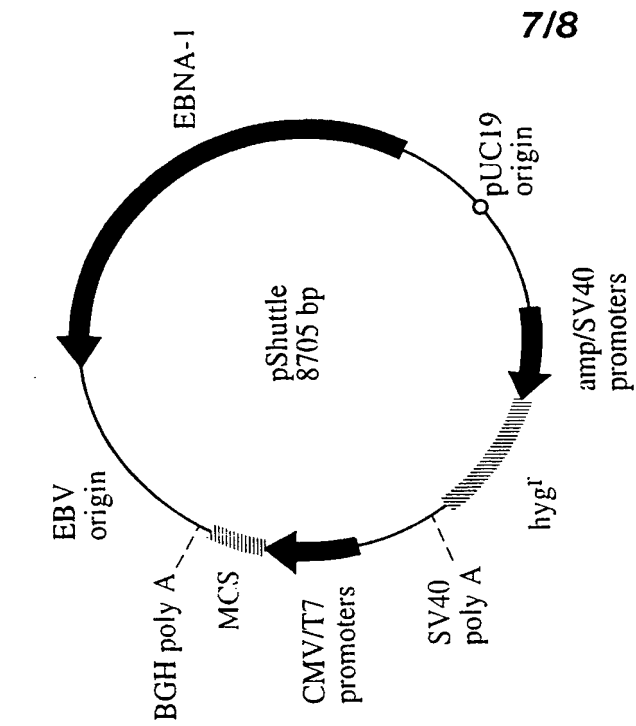
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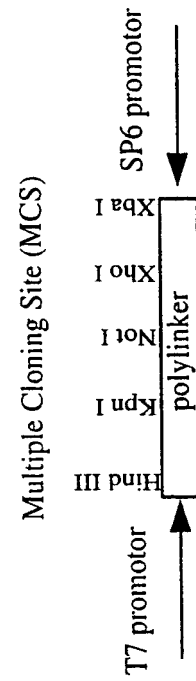
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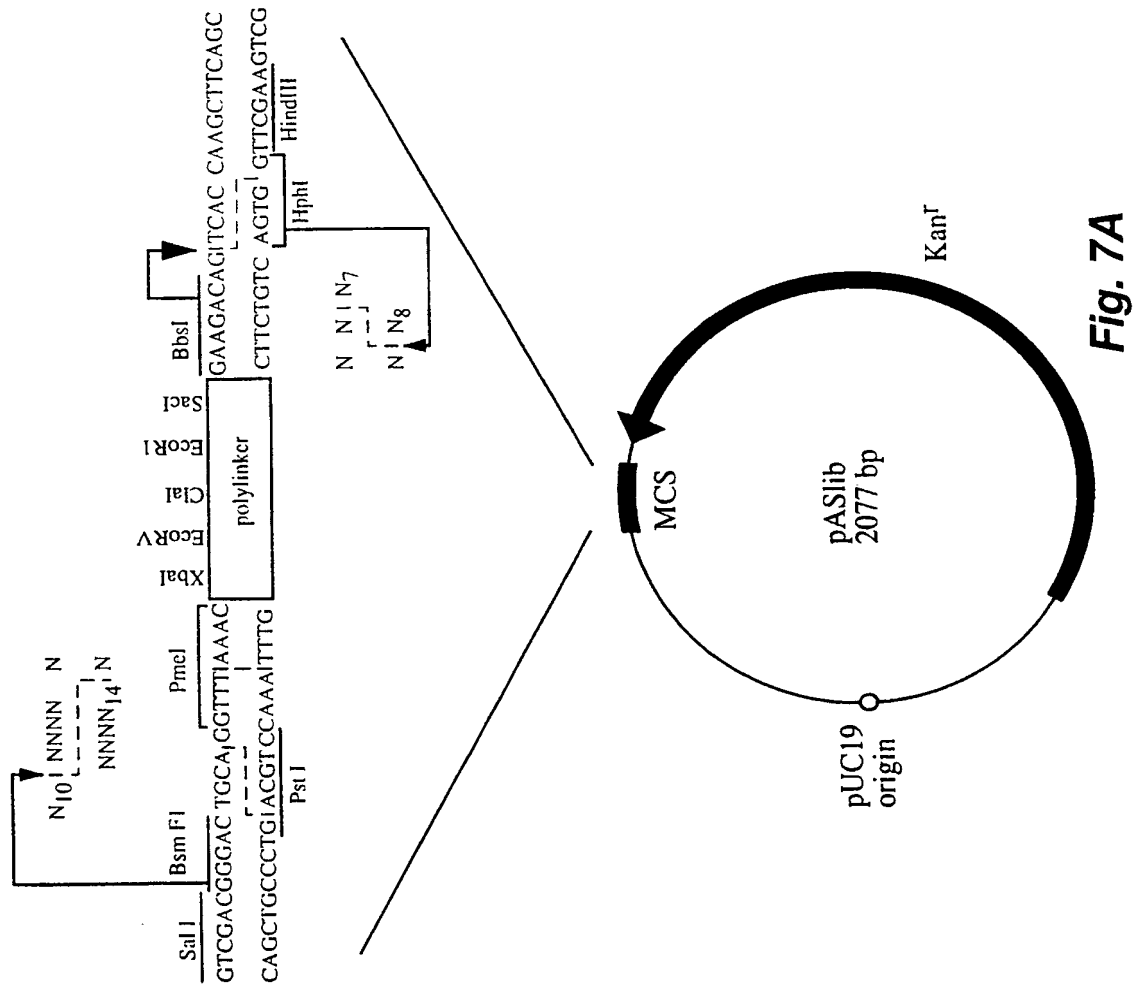
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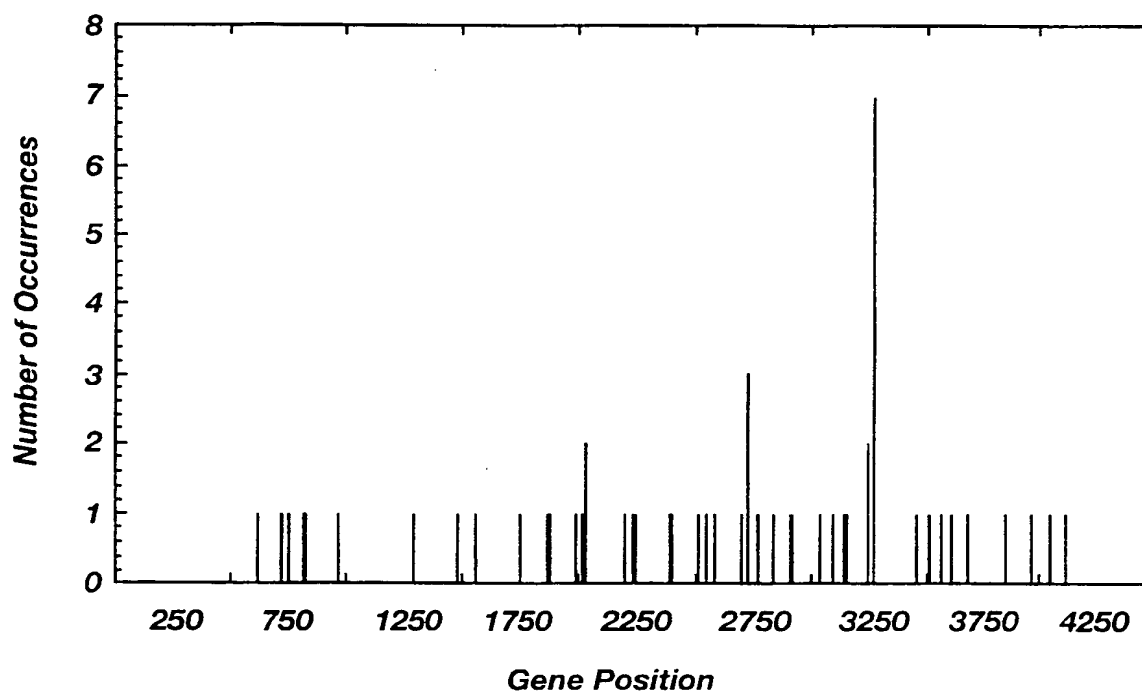
Fig. 8

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<110>   Ruffner, Duane E.
           Pierce, Michael L.
           Chen, Zhidong

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SUBSTITUTE SHEET (RULE 26)

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SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US99/06742

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68; C12N 15/00, 15/85; C07H 21/04

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.31, 320.1, 375; 536/23.1, 23.2, 24.3, 24.33, 24.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog: CAS, Medline, Biosis, Derwent Biotechnology Abstracts

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,496,698 A (DRAPER et al.) 5 March 1996, see entire patent.	1-21
A	US 5,525,468 A (MCSWIGGEN) 11 June 1996, see entire patent.	1-21
A	MATVEEVA et al. A rapid in vitro method fro obtaining RNA accessibility patterns for complementary DNA probes: correlation with an intracellualr pattern and known RNA structures. Nucleic Acids Research. 1997, Vol. 25, No. 24, pages 5010-5016, see entire document.	1-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	
"E"	earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	
"P"	document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 JULY 1999	19 JUL 1999

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	JOHN LE GUYADER
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A,P	PIERCE et al. Construction of a directed hammerhead ribozyme library: towards the identification of optimal target sites for antisense-mediated gene inhibition. Nucleic Acids Research. 1998, Vol. 26, No. 22, pages 5093-5101, see entire document.	1-21

Form PCT/ISA/210 (continuation of second sheet)(July 1992)★

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/6, 91.1, 91.31, 320.1, 375; 536/23.1, 23.2, 24.3, 24.33, 24.5

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